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THE USEFULNESS OF THE FLUORESCENT ANTIBODY TECHNIQUE IN STUDYING EXPERIMENTAL NEPHRITIDEDES. ITS POSSIBLE APPLICATIONS TO UNDERSTANDING THE PATHOGENESIS OF SOME HUMAN NEPHROPATHIES

[Following is the translation of an article by Beatrice Carrier Seegal, M. D., Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, based on research sponsored by the National Science Foundation Grant 2290 and Grant 4910 and by the National Institute of Health, Public Health Service Grant H 3929, in the Italian-language publication Rass. Fisiop. Cl. Ter., Vol XXXI, No 12, Dec. 1959, pages 1063-1078.]

Mister President, Ladies and Gentlemen:

I wish, first of all, to express my most sincere thanks to the illustrious Roman Academy of Medicine and Surgery in the person of its president, Professor Di Mattei, for having welcomed me as a guest at this meeting and for having given me the opportunity of speaking to you about some of our research work pertaining to the application of the fluorescent antibody technique in studying experimental and human nephritis.

Part of this work was suggested by an assistant in the Institute of Medical Pathology of the University of Rome, Doctor Andres, who -- under the direction of Professor Cassano -- visited our laboratory in the autumn of 1956. We are working at present in collaboration with Professor Fiaschi and Doctor Andres on the application of the fluorescent antibody technique to human material, and I propose to inform you as follows on this type of research.

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The subject of renal diseases -- that includes acute glomerulonephritis, chronic glomerulonephritis and the nephrotic syndrome -- creates numerous etiologic and pathogenic problems, still unsolved at the present time.

It has now been established that acute glomerulonephritis is usually preceded by a streptococcal infection and that the microorganism belongs most frequently to Type 12. In spite of that, the precise mechanism by means of which Streptococcus pyogenes provokes nephritis is still unknown.

On the other hand, in patients afflicted with chronic glomerulonephritis or with the nephrotic syndrome proof of a preceding infection with Streptococcus pyogenes and with other diseases, characterized by the presence of anti-streptococcic antibodies in the area, is often lacking.

It was written about fifteen years ago in an important treatise on medicine that although Richard Bright had been dead now for over 100 years, not much could be added to knowledge about his disease.

It is a fact that in the past, clinics were obliged to venture difficult probably diagnoses whenever they were confronted with nephropatients, and very often their diagnoses were proved to be wrong by autopsy findings.

Today a series of renal biopsies have opened up new possibilities for studying nephropathology. This technique gives access to the renal tissue in every phase of the disease and has made possible electron-microscopic research. To the above must be added the ability to reproduce various forms of nephropathies in animals and the perfection of the fluorescent antibody technique. All this shows that much progress has been made in the knowledge of the relationships occurring between pathogenesis and clinical conditions.

The clinical and histopathologic picture of acute nephritis, of chronic nephritis and of the nephrotic syndrome can be reproduced experimentally by means of an immunological technique consisting of the administration of specific antibodies for their renal tissue (Masugi's nephritis) to animals. It has been determined that the antigen-antibody reaction appears in the experimental animal within the renal antigen and the antirenal antibody that has been injected into it. It must be recalled, however, that experimental nephritis is the product of an allergic reaction in which the host itself provides the antigen instead of the antibody.

Nevertheless, it often happens that renal decompensation shows up only months and years after the injection of the nephrotoxic serum. The mechanism by means of which the kidney may incur lesions so serious that they cause death -- with a disease that appears to be capable to perpetuating itself autonomously -- has not yet been explained satisfactorily.

Recently a new technique was perfected that enables us to add new knowledge to the old ideas concerning the immunoallergic mechanisms of experimental nephropathy. It is to be hoped that this technique may also be useful in studying the pathogenesis of human nephropathies. We are referring to the fluorescent antibody technique of Coons and Kaplan.

Antibody proteins can be tied to or "labelled" with fluorescein isocyanate. The stained antibody is then used to dye a section of fresh renal tissue that has been sectioned while it is frozen. If the renal tissue contains a specific antigen for the antibody stained with fluorescein, it will fix the antibody in position and -- when the renal tissue is examined under the microscope with ultraviolet light, that causes fluorescein to emit a yellow-green light -- the areas in which the antibody has been fixed can be easily identified. In this way the seat of the specific antigen for the fluorescent antibody is determined.

Antibodies "labelled" with fluorescein have been used to identify the tissular seat of viruses, Rickettsia and bacteria, as well as the location of heterogeneous proteins of non-infective nature and of native proteins (produced by the host).

The fluorescent antibody technique, applied to the study of experimental nephritis, has provided an obvious proof in favor of the allergic nature of the disease. It has been possible to follow the distribution of the nephrotoxic serum in the organism of the nephritic animal, to prove that it is localized in the kidney and to establish that this serum remains in the tissues for a long period of time.

Antibodies stained with fluorescein have also been used to

study the altered distribution of globulins of an autochthonous nature in the nephritic animal. The increase in these globulins could indicate a location with an elective antibody concentration.

The series of slides that will now be projected will illustrate the application of the fluorescent antibody technique to the study of the pathogenesis of experimental nephritis. Research of this kind points out very clearly the usefulness of extending it to the study of human nephritis. Consequently — after discussing the experimental disease — I shall dwell on some subjects for study concerning human nephropathies on which work is being conducted at present, in close collaboration, in the Institute of Medical Pathology of the University of Rome and in the Department of Microbiology of the College of Physicians and Surgeons, Columbia University, New York.

But first of all let me thank some of my collaborators, among them Doctors Emily Loeb, Margaret Bevans, Evelyn Gaynor, Ruth Friedman and Henry Metzger. I am especially grateful to Dr. Konrad Hsu who took all the photographs that you shall see shortly. Mrs. Margo Hasson, Mrs. Mildred Rothenberg and Dr. Fred Urquhart have contributed much more to this research than they were obliged to do.

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The data pertaining to experimental nephritis will be presented in the form of three conclusive considerations, each of which will be accompanied by slides illustrating the results of the experiments that have enabled us to draw up the conclusions.

<u>The Production of Nephrotoxic Serum.</u>

Rabbits and ducks were used to produce nephrotoxic serums. The rabbits were immunized intraperitoneally with a 20% suspension in physiological solution of kidney or of other tissues from rats or dogs. The ducks were inoculated intramuscularly with rat or rabbit kidney or other organs mixed with Freund's adjuvants. These two methods of immunization yielded the best results in the preparation of nephrotoxic serums, although in some cases the immunization had to be protracted considerably. The placenta and the lung — along with the kidney — can stimulate the production of nephrotoxic or nephritis-causing antibodies. Blood serum, red corpuscles, the brain and the aorta were used experimentally as antigens, but they did not produce nephritogenic antibodies.

<u>Method for Revealing the Localization of the Nephrotoxic Serum by Means of Fluorescent Antibodies.</u>

First of all, antibodies must be prepared from nephrotoxic antibodies. For example, an antibody from nephrotoxic duck serum is obtained by precipitating, with sodium sulfate, the blood globulins of normal ducks. These globulins — mixed with Freund's adjuvants — are then injected into a rabbit. The antiglobulin duck antibodies are then precipitated by the serum of this rabbit and are "labelled" with fluorescein isocyanate, in accordance with Coons's technique. These "labelled" antiduck rabbit globulins can be used to demonstrate the presence of duck globulins in any animal tissue that has been injected with specific antiorgan antibodies produced in the duck. In other words, if a nephritic rat is put to

death after having been injected with rat antikidney duck serum, a fragment of its kidney can be frozen and sectioned in a cryostat into 4 micron segments. One section is put on a slide, stained with fluorescent duck antiglobulin antibodies. When the section is examined with the ultraviolet microscope, it can be observed that the basal glomerular membranes are fluorescent, because they emit a yellow-green light. They contain, in fact, nephrotoxic duck serum, and consequently they have fixed the antiduck globulins "labelled" with fluorescein. Nephrotoxic duck serum can thus be revealed in any part of the body. On the other hand, if the injected antiorgan serum has been prepared in a rabbit, the serum that produces fluorescence contains rabbit antiglobulin duck antibodies.

By using this fluorescent antibody technique, three facts concerning experimental nephritis were established.

The first fact is that for the appearance of glomerulonephritis it is necessary to localize the nephrotoxic serum in the glomeruli.

Antikidney rat serum obtained from rabbits and ducks causes proteinuria in rats and a histologic picture of glomerulonephritis when it is inoculated in them intravenously. It can be demonstrated that the nephrotoxic serum is localized in the glomeruli — and especially in the basal membranes — staining a kidney section with specific fluorescent antibodies for rabbit and duck globulins. This is illustrated in Figure 1, showing a kidney section from a rat put to death 24 hours after receiving an injection of rat antikidney duck serum. The section was stained with duck antiglobulin antibodies "labelled" with fluorescein. The basal glomerular membranes show up flourescent, because the nephrotoxic serum localized in these areas has fixed the specific antibody "labelled" with fluorescein.

Other nephritogenic antisera — and especially rat antilung and antiplacenta serums — are also localized in the basal glomerular membranes.

Some of the nephrotoxic serums that were injected can be demonstrated in two other organs: the spleen and the adrenals. In the spleen the serum is localized in the red pulp cells. In the adrenals it is found in the endothelial cells of the cortical capillaries. In each of these areas the concentration of heterogenous globulins is approximately equal to 10% of the glomeruli concentration.

Neither rat antilung serum nor rat antikidney serum has been found in significant amounts in the skeletal muscles, in the myocardium, in the liver, in the pancreas or in the lungs.

Antiorgan serums that do not provoke nephritis either are not localized in the glomeruli in a sufficient amount or are present for short periods of time.

Rabbits inoculated with rabbit antikidney serum and dogs inoculated either with dog antikidney serum or dog antiplacenta serum also demonstrate localisation of the nephrotoxic serum in the basal glomerular membranes. Figure 2 illustrates the localisation of duck nephrotoxic serum in the kidney of a rabbit inoculated six days earlier with rabbit antikidney duck serum. In this case also the fluorescent antibody used to stain the section is specific for duck globulins.

Thus it is evident that the nephrotoxic serum is localised almost exclusively in the basal membranes of the glomerular capillaries. This is true whether antikidney serum, antilung serum or

antiplacenta serum is injected, whether the antiserum is produced by rabbits or by ducks, and, finally, whether a rat, a dog or a rabbit is used as experimental animal.

The second fact established by means of the fluorescent antibody technique is that the nephrotoxic serum remains fixed in the basal glomerular membranes for many months.

Kidneys removed from nephritic rats at time intervals that extended to nine and one-half months after the disease began still contained part of the nephrotoxic serum that had been injected. Figure 3 shows a section of a kidney of a rat afflicted with chronic nephritis, 192 days after the injection of rat antikidney rabbit serum. The preparation is stained with rabbit antiglobulin duck antibodies that have been "labelled" with fluorescein. The weak fluorescence of the glomeruli indicates the persistence of the nephrotoxic serum in these areas.

Nephritic rabbits were examined for about four months and nephritic dogs for about six months after the injection of nephritis-producing serums. In each case these serums were still demonstrable in the glomeruli.

It has not yet been determined precisely how long the nephrotoxic serum remains in the glomeruli. It was still detectable in rats after nine and one-half months, in dogs after six months, and in rabbits after four months.

The third observation made possible by the fluorescent antibody technique is that when an animal is injected with nephrotoxic serum, an increase in the amount of "native" globulins is observed in the kidney.

This was ascertained by staining tissue sections from nephritic animals with rat antiglobulin antibodies "labelled" with fluorescein and, respectively, with dog or rabbit antiglobulin antibodies, also "labelled" with fluorescin. In this way, there was found a moderate increase in the globulins in rat and dog kidney and a much more pronounced increase in the rabbit kidney.

Figure 4 is a control section of kidney from a normal rat, stained with rat antiglobulin rabbit globulins "labelled" with fluorescein. A weak fluorescences is observed in the endothelial cells of the peritubular capillaries and in the glomeruli. This shows a characteristic picture of normal tissue stained with "native" antiglobulin antibodies and it could also occur in dogs and rabbits.

Two or three days after the injection of the nephrotoxic serum, the amount of globulins capable of being stained with antiglobulin serum increased. Figures 5 and 6 illustrate the presence of "native" globulins in the glomeruli of nephritic rats and rabbits. Figure 5 is a kidney section from a rat injected six days previously with rat antikidney rabbit serum and stained with rat antiglobulin rabbit antibodies "labelled with fluorescein. The presence of an increased amount of rat globulins in the glomeruli is demonstrated by the increase in the fluorescence that is found localized especially in the cells (See Fig. 4). Figure 6 shows a section of rabbit kidney 30 days after the injection of nephrotoxic serum. Great amounts of "native" globulins are found there, apparently localized predominantly in the basal membranes.

The significance of these experiments is constituted by the fact that both the antigen (nephrotoxic serum) and the possible antibody (globulin of autochthonous origin) coexist in the renal tissue for several months. This corroborates the theory that in experi-

GRAPHIC NOT REPRODUCIBLE

Fig. 1. Section of rat SDC 100 kidney one day after the injection of rat antikidney duck serum. Stained with duck antiglobulin rabbit serum globulins, labelled with fluorescein isocyanate. Note that the fluorescence is apparently limited to the basal glomerular membranes, which proves the localisation of the nephrotoxic serum in these structures of the rat kidney.

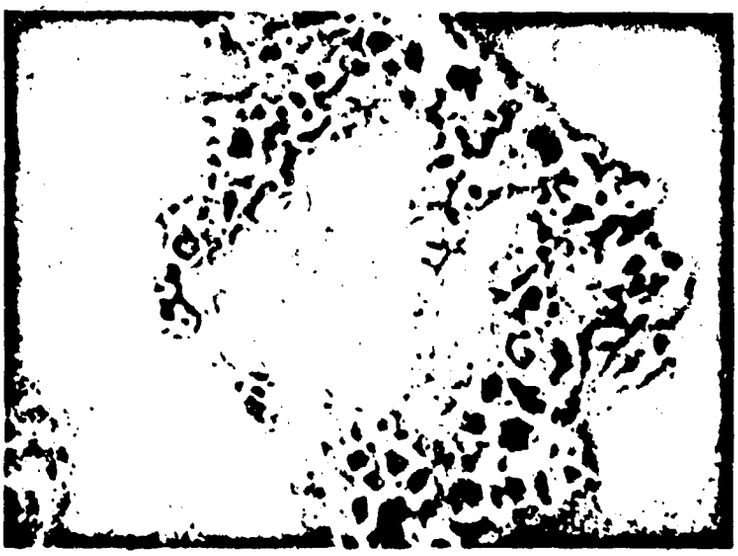

Fig. 2. Section of rabbit 261 kidney, six days after injection of rabbit antikidney duck serum. Stained with globulins obtained from duck antiglobulin rabbit serum, labelled with fluorescein. The specific nephrotoxic serum is apparently localised in the basal glomerular membranes of the rabbit.

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Fig. 3. Section of rat SDB 806 kidney 192 days after injection of rat antikidney rabbit serum. Stained with labelled globulins obtained from rabbit antiglobulin duck serum. Note that in this rat, afflicted with chronic nephritis, nephrotoxic serum is found in the glomeruli. Note also the autofluorescence in the artery (top left) and in the tubules (bottom left).

Fig. 4, Section of normal rat kidney stained with fluorescent rat antiglobulin rabbit serum. The weak fluorescence observed in the peritubular endothelial cells and in the glomeruli indicates the presence of "native" globulins in these cells.

Fig. 5. Section of rat SDC 205 kidney six days after injection of rat antiglobulin rabbit serum. The preparation is stained with globulins of rat antiglobulin rabbit serum labelled with fluorescein. The amount of "native" globulins present in the endothelial cells has increased.

Fig. 6. Renal section of rabbit 280, 30 days after injection of rabbit antikidney duck serum. The preparation is stained with fluorescent globulins of rabbit antiglobulin duck serum. The "native" globulins are clearly localized in the basal glomerular membranes.

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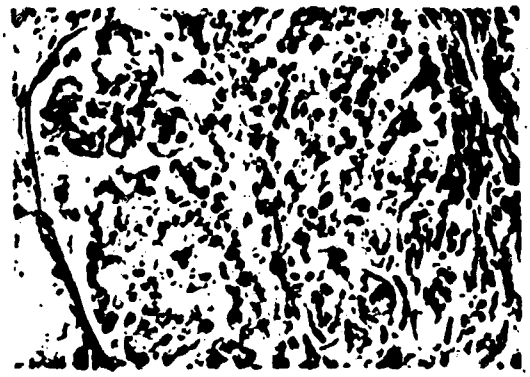
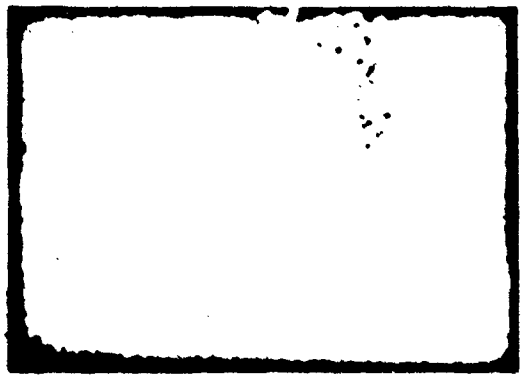


a. Chronic glomerulonephritis; acute focal glomerulitis. Ritter-Olson. 300 X

b. Fluorescence emanated from the glomerulus after contact with antistreptococcic serum labeled with fluorescein isocyanate. 300 X



c. Human antigamma globulin serum labelled with fluorescein isocyanate induces elective fluorescence in the glomerulus. 450 X

Fig. 7. A. M., 36 years old. Recurring acuteness in chronic glomerulonephritis. Antioxygen streptolysin, 833 units per cc.

GRAPHIC NOT REPRODUCIBLE

a.

Membranous glomerulitis and glomerular and periglomerular hyalinosis. Hotchkiss-McManus. 300 X

b.

After contact with antistreptococcic serum labelled with fluorescein isocyanate, the capillary ansae of the glomerulus are clearly outlined. 727 X

c.

With labelled antigamma globulin serum, the fluorescence appears localised in the glomerular capillary ansae. 343 X

Fig. 8. B. C., 36 years old. Chronic glomerulonephritis. Nephrotic syndrome. Antioxygen streptolysin, 250 units per cc.

mental nephritis the inflammatory renal disease, persisting for a long time after the nephrotoxic serum has reached the glomerulus, can be supported by an antigen-antibody reaction.

This antigen-antibody reaction can be caused by residues of the nephrotoxic serum reacting with the glomerular proteins or by residues of the nephrotoxic serum reacting with the antibody produced by the host in the presence of the heterogeneous antigen, or by both.

At any rate, these experiments have confirmed that chronic renal lesions can be the result of an allergic type of inflammation.

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Therefore, how can the fluorescent antibody technique be used today to demonstrate a possible heterogeneous antigen and a "native" globulin — containing antibodies of that antigen — in the renal tissue of nephritic individuals?

When Dr. Andres was in New York at the Presbyterian Columbia Medical Center we discussed this problem.

The only sure information concerning the etiological agent of this group of renal diseases is constituted by the fact that an infection by hemolytic streptococcus precedes the majority of the cases of acute nephritis. Therefore, it seems logical to explore the possibility that some antigens — produced by the streptococcus — may be found in the kidney in case of disease.

The second point deserving study is whether human gamma globulins — vectors of antibodies — are present in the kidney in greater amounts than the ones found in any tissue subjected to phlogistic processes.

Serious technical difficulties have slowed our progress considerably. Both the first antistreptococcic serum, prepared for these experiments, and the first antigamma globulin serum did not yield satisfactory results. But Professor Fiaschi and Dr. Andres, who sent me by airmail fragments of frozen renal tissue, have been very patient, and at least some encouraging results have come from the preliminary study of the last 18 cases that were sent to us.

In this group — that includes a fragment of normal renal tissue and a fragment of pyelonephritic kidney together with other bioptic fragments of acute, subacute and chronic nephritis, with or without nephrotic syndrome — we were able to demonstrate that the renal tissue of four patients responded positively to the antistreptococcic serum. One of these belonged to an observation of acute nephritis, two of subacute nephritis and one to a recurring acuteness of chronic nephritis apparently induced by streptococcic infection of the upper airways.

Figure 7 illustrates this last case. The tissue is stained with fluorescent antistreptococcic serum. In this one, as in the other three renal biopsies in which antistreptococcic serum was found, the antigen appeared to be contained more in the glomerular cells than in the basal membranes.

Not all the sections have been studied up until now with antiglobulin serum of tested efficacy. Nevertheless, an increase in the gamma globulins in the glomeruli of five of the ten kidney fragments examined was demonstrated. This can be observed in Figure 8.

The renal tissue of these same patients has obviously been

studied by Professor Fiaschi and by his collaborators, using ultrathin histologic preparations and -- in some cases -- the electron microscope.

Although our attempts to add new information to the present knowledge on the localisation of antigens and antibodies in the renal glomeruli by using the fluorescence technique are still in their initial phases and no conclusion may, therefore, be drawn, I strongly hope, nevertheless, that useful progress may be derived from the joint experiments. For this reason I am very happy to be associated with the group of students, under the guidance of Professor Cassano, engaged in this intensely interesting work program.

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